INHIBITING GLYCOLYSIS ENHANCES T FOLLICULAR HELPER CELL DIFFERENTIATION AND SURVIVAL UPON HUMAN IMMUNODEFICIENCY VIRUS INFECTION

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DEDICATION

I dedicate this work to my father- Shirish Balkrishna Rane who is a constant source of inspiration to me.

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INHIBITING GLYCOLYSIS ENHANCES T FOLLICULAR HELPER CELL DIFFERENTIATION AND SURVIVAL UPON HUMAN IMMUNODEFICIENCY VIRUS INFECTION

Human immunodeficiency virus (HIV) primarily infects T helper (Th) cells. Decrease in the number of Th cells is the hallmark of HIV infection. Latent reservoirs of human immunodeficiency virus (HIV) are the leading barrier towards eradication of HIV infection. T Follicular helper (Tfh) cells are a subset of Th cells that function to provide aid to B cells for their maturation, affinity selection and antibody class switch. Several studies have shown that Tfh cells are a major reservoir of latent as well as productive hiv infection. But in contrast to the fate of other Th cell subsets, the frequency of Tfh cells was shown to have increased during HIV infection which could not be attributed to their reduced susceptibility to HIV infection.

The hypothesis was that Tfh cells possess a unique metabolic phenotype that protects them from HIV induced cell death. Transcriptome analysis of Th subsets from human donors and showed that Tfh cells rely less on glycolysis for their energetic requirements and instead have increased transcription of fatty acid synthesis genes. This finding was corroborated by seahorse extracellular flux assay. The results shoId that glycolysis was not essential for Tfh cell differentiation in-vitro. The observed increase in Tfh cell frequency could not be attributed to increased Tfh differentiation upon HIV infection since HIV infection inhibited the differentiation of both non-Tfh and Tfh cells. The results found that bypassing the glycolytic pathway by providing Tfh cells with Galactose in the medium protected ex-vivo infected primary tonsillar cells from HIV

induced cell death. This protection could be partly explained by the induction of Baculovirus IAP repeat containing 5 (BIRC5) when the cells utilized Galactose instead of Glucose. The studies together show that Tfh cells have an oxidative metabolic phenotype which protects them from HIV induced cell death in part by induction of BIRC5 expression.

Quigui (Andy) Yu, MD, PhD, Chair

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2DG	2’ deoxy Glucose
AIDS	Acquired Immunodeficiency Syndrome
ART	Anti-retroviral therapy
ATP	Adenosine Triphosphate
AZT	Zidovudine
Bcl6	B cell lymphoma 6
BIRC5	Baculoviral IAP repeat containing 5
Blimp1	B- lymphocyte induced maturation protein 1
CCR	C-C motif chemokine receptor
CD	Cluster of differentiation
CDC	Centers for disease control and prevention
cDNA	complementary deoxyribonucleic acid
CXCL	C-X-C motif ligand
CXCR	C-X-C motif chemokine receptor
DNA	Deoxyribose nucleic acid
ECAR	Extracellular acidification rate
ELISA	enzyme linked immunosorbent assay
Env	envelope
ETC	electron transport chain
FAO	fatty acid oxidation
FBS	fetal bovine serum
FCCP	carbonyl cyanide 4- (trifluoromethoxy) phenylhydrazone
FoxP3	Forkhead box P3
FVD	fixable viability dye
GC	germinal center
HAART	Highly active antiretroviral therapy
HIV	Human immunodeficiency virus
ICOS	inducible T cell co-stimulator
ICOSL	inducible T cell costimulatory ligand
IFN-γ	interferon gamma
Ig	immunoglobulin
IL	interleukin
OCR	oxygen consumption rate
PBMCs	Peripheral blood mononuclear cells
PBS	phosphate buffered saline
PD	programmed death
RPMI	Roswell Park memorial institute
TNFRSF4	Tumor necrosis factor receptor superfamily member 4
YM155	1-(2-Methoxyethyl)-2-methyl-4, 9-dioxo-3-(pyrazin-2-methyl)-4, 9- Dihydro 1-H naphtho imidazolium bromide

Introduction

1.1 Human immunodeficiency virus infection

In 1981, the Centers for Disease Control and Prevention (CDC) reported 5 cases of advanced unexplained immune-deficiency in homosexual men (1). This disease was termed as acquired immune-deficiency syndrome (AIDS). In 1983, two independent groups isolated the causative retrovirus which was coined as the human immunodeficiency virus type 1 (HIV-1, hereafter referred to as HIV) (2, 3). The HIV pandemic has claimed over 39 million lives, with approximately 37 million people worldwide living with HIV/AIDS (4), and will continue to contribute to human morbidity and mortality as there is no vaccine or curative treatment available. Although antiretroviral therapy (ART) is effective in controlling viral replication, the global prevalence of ART is only 59% (5). It is impossible to eliminate the HIV epidemic without further advancements in biomedical research of HIV infection and therapy.

1.2. HIV life-cycle

HIV preferentially infects activated CD4 T cells. All subtypes of CD4 T cells including T follicular helper (Tfh) cells are susceptible to HIV infection. HIV envelope (Env) protein binds to CD4 molecule on the target cell surface and undergoes conformational change. This conformational change allows Env to bind to one of the two chemokine receptors- C-X-C chemokine receptor 4 (CXCR4) or C-C chemokine receptor 5 (CCR5). The virus enters the cells by fusion of the cell membrane and viral Env. Uncoating of viral capsid releases the pre-integration complex in the cytoplasm. Nuclear transport machinery then transports the pre-integration complex to the nucleus. Reverse transcription yields double-stranded complimentary viral DNA, one and two long

terminal repeat (LTR) containing circles and linear forms. The linear forms integrate into the host genome to become provirus. Cellular activation induces transcription from the provirus. Viral regulator of virion gene expression (Rev) protein transports intron-containing viral RNAs to the cytoplasm from the nucleus. Viral structural and accessory proteins are generated. After virion assembly, the progeny virus is released from the infected cells via budding from the cell membrane (Figure 1).

Figure 1

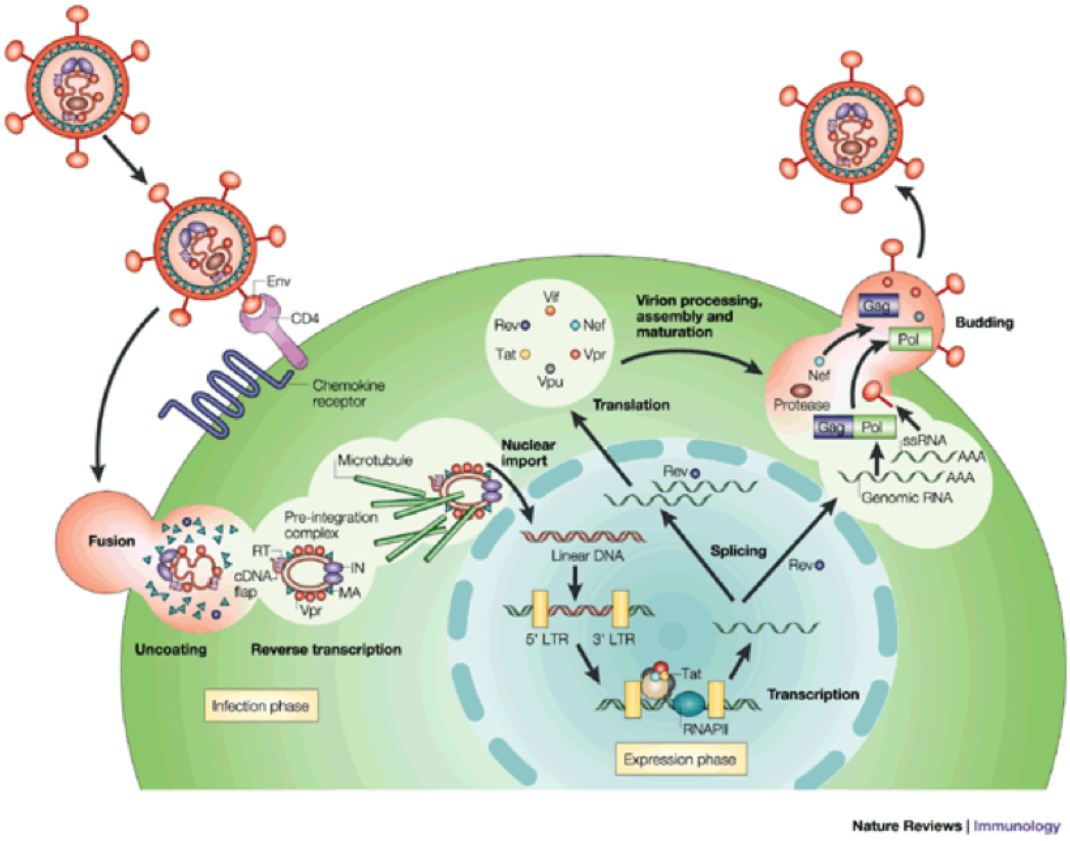


Figure 1. HIV life-cycle

HIV primarily infects CD4 T cells. Engagement of viral Env with CD4 causes conformational changes in the virus and allows binding to the coreceptors- CXCR4 or CCR5. Upon entry into the host cell, the viral reverse transcriptase converts the viral RNA to double stranded DNA. The pre-integration complex consists of the viral dsDNA, the viral integrase protein and other viral and host proteins. The pre-integration complex is transported into the host cell nucleus. The viral integrase integrated the HIV DNA into

the host genome. Viral trans-activator of transcription (Tat) facilitates transcription from the HIV promoter.

1.3 HIV infection and cellular metabolism

Viruses are dependent on the host for provision of bioenergetics resources for the completion of their life cycle. Several studies have shown that viruses like hepatitis C virus (HCV), herpes simplex virus (HSV) and human cytomegalovirus (HCMV) alter host cell metabolism (6, 7). HIV replicates most efficiently in activated CD4 T cells. Naïve or resting CD4 T cells are resistant to HIV infection due to their inability to support reverse transcription as they have low reserves of nucleotides (8). Studies have shown that HIV infection modulates its target cells. Metabolite analysis of HIV-infected T cells and macrophages found that HIV infected-T cells had upregulated glycolysis. HIV infected macrophages in contrast had reduced Glucose uptake and glycolysis intermediates (9). Williamson et al demonstrated that Glucose transporters and hexokinase expression in T cells was dependent on viral replication and not on viral proteins (10). Hegedus et al showed that HIV replication and virion production requires glycolysis (11). Huthoff group observed that glutamine concentration was increased in HIV infected cells. The increased glutamine levels were redirected for the production of nucleotides (12). Thus, HIV life cycle is intimately dependent on the metabolism of the infected host cell.

1.4 Discovery of Tfh cells

The role of thymus in antibody immune response was demonstrated in 1965 by Miller (13). Thymic cells rather than the thymic niche or thymus-derived factors were critical for antibody producing cells(14). While studying antibody responses against hapten conjugated to protein carriers, Mitchison et al showed that T cells specific for the protein carrier provided help for maturation of hapten specific antibody producing B cells

(15). These studies together cemented the role of T cells in antibody response. Sprent showed that T-B cell interaction was major histocompatibility complex (MHC) class II restricted (16). Studies using either antigen-presenting cells (APCs) or B cells lacking MHC class II molecules confirmed that T cell help to B cells requires T cell recognition of peptide-MHC II complexes(17). Several groups demonstrated that T helper cells expressing the CXC chemokine receptor 5 (CXCR5) localized to B cell follicles and provided help for B cell maturation (18-20). These studies first coined the term T follicular B helper (Tfh) cells.

1.5 Phenotype of human Tfh cells

Three studies independently showed that B-cell lymphoma 6 (Bcl6) is an essential transcription factor for Tfh cell differentiation but not essential for differentiation of other T helper cell subsets (21-23). Gene expression studies of human Tfh cells revealed that several genes including Interleukin -21 (IL-21), programmed death-1 (PD-1), CXCR5, Bcl6, and inducible T cell co-stimulator (ICOS) were upregulated in Tfh cells.

Figure 2

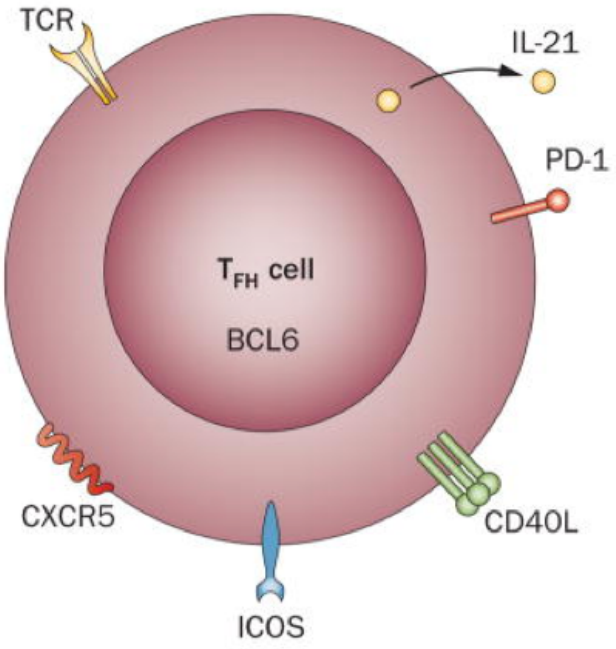


Figure 2. Phenotype of human Tfh cells

Tfh cells are defined as CD4 T cells expressing surface markers CXCR5, PD-1 and ICOS, the transcription factor Bcl6 and secretion of the cytokine IL-21.

(Craft JE, 2012 Follicular helper T cells in immunity and systemic autoimmunity, Nat.Rev. Rheum, 8.6:337-358)

1.6 Tfh cell differentiation

Differentiation of Tfh cells is a multistage multi-factorial process. The multi-signal process of Tfh differentiation accommodates heterogeneity. The process of Tfh cell differentiation can be divided into three stages-priming, commitment to Tfh cell fate at the B and T cell border of the germinal center (GC) and full polarization. The differentiation process starts by initial priming of naïve T cells by dendritic cells (DCs). Human DCs have been shown to induce differentiation of IL-21-producing Tfh-like cells that are capable of inducing antibody production by B cells. DCs induced differentiation of IL-21-producing Tfh cells through IL-12. Bcl6 expression is upregulated after TCR activation. Early induction of Bcl6 was observed as soon as 2-4 days in the absence of B cells. However, there was a significant reduction in Tfh differentiation by day 8 in absence of B cells. This indicates that even though DCs are able to transiently induce early differentiation of Tfh cells, antigen presentation by B cells is important to sustain Tfh differentiation in vivo.

Extensive studies in humans have shown that multiple cytokines contribute towards Tfh differentiation – mainly TGF-β, IL-12 and IL-23 (24). Activated human CD4 T cells cultured in the presence of IL-12 or IL-23 showed increased expression of the Tfh markers - CXCR5, ICOS and Bcl6. In addition, the combination of the cytokines IL-12 and TGF- β induced the highest expression of Tfh markers on activated CD4 T cells. Activin A, a member of the TGF-β superfamily, in combination with IL-12 was also shown to drive Tfh cell differentiation in vitro (25).

The migration of Tfh cells to B cell follicles enables the critical step of B cell dependent stage of Tfh differentiation. Interaction with ICOSL expressed on activated B

cells is essential for Tfh cell differentiation. ICOS induced phospho-inositol 3 kinase (PI3K) activation regulated expression of Tfh derived effectors like IL-21 and CD40.

Two phenotypically well-defined populations of Bcl6-expressing Tfh cells are identified in mice as well as humans. These are referred to as Tfh and GC Tfh cells. GC Tfh cells are further differentiated Tfh cells that express the highest levels of Bcl6 and CXCR5 in human tonsillar CD4 T cells. It is currently unclear whether GC Tfh are the terminally differentiated cells or whether they can alternate between the GC Tfh and Tfh phenotypes.

1.7 Functions of Tfh cells

Tfh cells provide help for B cell maturation and affinity maturation . Tfh cells are essential for the formation and maintenance of GCs, for generation of memory B cells and plasma cells.CD40L is highly expressed on activated T cells, while CD40 is a co-stimulatory protein found on the surface of APCs. CD40-CD40 ligand (CD40L) signaling is important for sustenance of GC B cells. CD40-CD40L interaction provides survival signals to the highly apoptotic GC B cells.

PD-1 is an inhibitory receptor for T cells. Interaction of PD-1 with its ligands PD-L1 or PD-L2 prevents T cells from killing the target cells. Recent studies have shown that PD-1 drives the localization of Tfh cells in the B cell follicles of the GCs (26). Studies with PD-1 deficient mice have shown that PD-1 is critical for maintenance of GC B cells. In PD-1 deficient mice, lesser number of long-lived plasma cells were observed. PD-1 deficiency also resulted in reduced expression of IL-21 and IL-4 (27).

ICOS deficiency in humans resulted in severe reduction of memory B cells and abrogated antigen specific IgG response to vaccines (28).

IL-4 was shown to be highly expressed in GC Tfh cells independent of Th2 differentiation (29, 30). IL-4 provides anti-apoptotic signals to B cells inducing expression of the anti-apoptotic Bcl2 (31).

IL-21 drives plasma cell differentiation. IL-21 induces expression of the master transcription factor of plasma cells - Blimp1 via STAT3 (32).

Humans Tfh cells express high levels of CXCL13, the ligand for CXCR5. CXCL13 may enhance the recruitment of B cells to the GCs (33).

Figure 3

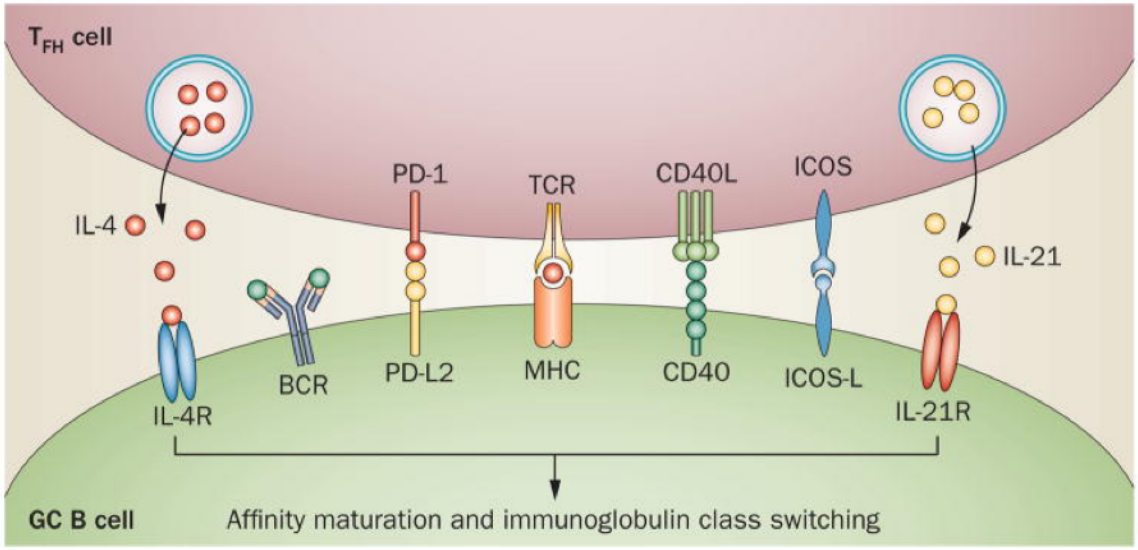


Figure 3. Function of Tfh cells

Tfh cells provide help to B cells for B cell affinity maturation and antibody class switching by cell-cell interactions via cell membrane factors like CD40-CD40L, ICOS and PD-1 and also through soluble effectors like IL-4 and IL-21.

(Craft JE, 2012 Follicular helper T cells in immunity and systemic autoimmunity, Nat.Rev. Rheum, 8.6:337-358)

1.8 Tonsillar Tfh cells vs blood CXCR5$^+$ T helper cells

Tissue Tfh cells have an activated T cell phenotype (CD45RO-) whereas CXCR5$^+$ T helper cells in the periphery have a memory T cell phenotype CD45RO$^+$. One hypothesis to explain this phenomenon states that peripheral CXCR5$^+$ Th cells are memory Tfh cells. However, a study using gene expression profiling found that there were significant differences in the gene expression profiles of the two populations (34). The study found differences in expression of several functional families of genes including transcription factors, cytokines and co-stimulatory molecules. Tonsillar Tfh cells have higher expression of Notch and Wnt signaling molecules compared to peripheral blood Tfh cells. IL-7 receptor and IL-10Rα were highly expressed on peripheral blood Tfh cells but downregulated in tonsillar Tfh cells (34)

1.9 Tfh cell dynamics during HIV infection

Soon after the discovery of HIV-1, studies have shown that GCs act as reservoirs of HIV-1 (35, 36). Like other T helper cell subsets, Tfh cells are susceptible to HIV-1 infection and support production of HIV-1 virions (35). Follicular DCs are thought to be responsible for entrapment of HIV-1 and spread to Tfh cells (37). Perreau et al showed that CXCR5$^+$PD-1$^+$ and CXCR5$^-$PD-1$^+$ cells isolated from chronically HIV-1 infected patients were enriched in HIV-1 specific CD4 T cells. They also showed that the Tfh population was the most efficient in supporting HIV-1 replication and virion production. In addition, they also observed that replication-competent HIV-1 could be isolated from Tfh cells from patients on ART with low viremia (<1,000 HIV-1 RNA copies) (38). Therefore, they concluded that Tfh cells act as a major reservoir of HIV-1.

Tfh cell frequency was also shown to expand during HIV-1 infection even after ART (39). Hong et al enumerated Tfh cells per unit area of lymph nodes of SIV infected macaques and observed approximately 4-fold increase in absolute Tfh cell numbers (40). This study indicates that during chronic HIV infection there is an increase in absolute numbers of Tfh cells instead of a slower decline in cell number. This observation is in contrast to the studies that show that HIV-1 infection causes massive loss of infected cells due to cell death. The decrease in total CD4 count is the hallmark of HIV-1 infection.

Chronic HIV-1 infection also leads to severe dysregulation of B cell immune response. In healthy individuals, most B cells in the periphery have naïve or memory B cell phenotype. In HIV-1 infected individuals, alterations in the B cell phenotypes are observed in peripheral blood. B cell subpopulations including immature transitional B cells, activated mature B cells and plasmablasts are observed at a higher frequency in HIV-1 infected patients compared to healthy individuals (41). HIV infection induced immune cell activation is a hallmark of HIV pathogenesis. B cell hyper-activation during HIV infection is characterized by hypergammaglobulinemia (42). HIV infection directly or indirectly causes B cell activation. HIV proteins such as gp120 and Nef have been proposed to affect B cell activation. Binding of gp120 to C type lectins on B cells induces antibody class switching (43). By inducing infected macrophages to produce pro-inflammatory cytokines, Nef drives polyclonal B cell activation (44). HIV infection also directly affects B cell activation by affecting Tfh cell function. Cubas et al observed that the frequency of PD-L1^{+} GC B cells in HIV-infected individuals was higher compared to uninfected individuals. Engagement of PD-1 on Tfh cells with PD-L1 on B cells resulted in reduced expression of ICOS and IL-21 (45).

1.10 Metabolism in immune cells

Immune response against environmental stress requires cell proliferation and secretion of effector molecules such as cytokines, chemokines and inflammatory mediators. This process is bio-energetically expensive and requires cells to adapt by switching their metabolic programming. All cells produce adenosine triphosphate (ATP) to maintain their basic cellular functions. The bioenergetics needs of cells are fulfilled by interconnected pathways of glycolysis, tricarboxylic acid (TCA) cycle and oxidative phosphorylation (OXPHOS). Glycolysis is an anaerobic process that occurs in the cytoplasm. Cells uptake Glucose from the environment through Glucose transporters. Once entered into the cells, Glucose is phosphorylated to Glucose 6-phosphate by hexokinases. Sequential glycolytic process yields two molecules each of pyruvate, NADH and ATP. Glucose 6-phosphate produced during glycolysis can enter the pentose phosphate pathway (PPP) that generates riboses which are the building blocks of RNA and DNA.

Under normoxic conditions, pyruvate is oxidized to yield ATP and NADH via the TCA cycle. In hypoxic conditions, pyruvate is reduced to lactate and NAD^+. NADH produced via the TCA cycle acts as a reducing agent in the oxidative phosphorylation pathway to generate ATP. During oxidative phosphorylation, sequential electron transfer in redox reactions releases energy which is utilized to produce ATP. Carbohydrates, amino acids and fatty acids can enter the TCA cycle by their conversion into acetyl-coenzyme A. Complete oxidation of Glucose by the TCA cycle and OXPHOS yields approximately 30-36 ATP molecules. Thus, in aerobic conditions, oxidative metabolism is a bioenergetically favorable pathway for ATP production.

Glycolysis and TCA cycle also provide intermediates for the biosynthesis of riboses, fatty acids and non-essential amino acids. Some tumor cells display preferential reliance on glycolysis in aerobic conditions even though it is energetically unfavorable. This process of aerobic glycolysis is known as the Warburg effect. Some tumor cells use Glucose to suppress OXPHOS. This event is known as the "Crabtree effect".

Adaptive immune cells have the unique features of antigen-specific proliferation to establish and maintain a pool of antigen specific memory cells. Antigen driven proliferation of adaptive immune cells requires a metabolic phenotype that provides for the synthesis of DNA, proteins, membranes and organelles. The quiescent memory cells require a metabolic phenotype that supports their long-term survival.

The energy needs of naïve CD4 T cells are fulfilled by oxidation of Glucose and fatty acids (46). Stimulated T cells proliferate in response to the antigen and produce effector molecules like cytokines. The metabolic requirements of T cells increase dramatically upon activation. Activated T cells fulfill this demand by increasing glutamine and Glucose metabolism while suppressing fatty acid oxidation. Studies have shown that glycolysis is upregulated in mitogen-stimulated peripheral T lymphocytes (47). Nutrient insufficiency prevents T cell activation. Jacobs et al show that Glucose metabolism induced by CD28 signaling is essential for T cell activation (48). The metabolic programming of activated T cells is regulated at the transcriptional and post-transcriptional stage. Activated CD4 T cells differentiate into distinct subsets based on the cytokine milieu. The metabolic phenotype of T effector subsets – Th1, Th2, Th17 and regulatory T (Treg) cells is well characterized. Studies have shown that the kinase mammalian target of rapamycin (mTOR) plays crucial role in T helper cell

differentiation. mTOR forms two complexes-rapamycin sensitive mTORC1 and the rapamycin insensitive mTORC2. mTORC1 signaling upregulates aerobic glycolysis in T cells. mTOR deficient T cells are able to differentiate only into Treg subset (49). Inhibiting mTORC1 signaling suppresses Th1 and Th17 differentiation but does not affect Th2 differentiation. In contrast, inhibiting mTORC2 signaling inhibits Th17 differentiation but not Th1 or Th17. In-vitro differentiated Th1, Th2 and Th17 cells exhibit increased glycolysis compared to naïve T cells whereas Tregs display elevated rates of lipid oxidation (50).

Studies in mice have shown that mTORC1 and mTORC2 induced glycolysis is sufficient to induce Tfh cell differentiation (51). In contrast, Ray et al have shown that Tfh cells display reduced glycolysis and mitochondrial respiration as a result of reduced mTORC1 activity when compared to Th1 cells (52). IL-2 induced mTORC1 activity and increased glycolysis was sufficient to drive Th differentiation away from Tfh lineage. Oestrich et al showed that Bcl6 binds to the promoters of glycolysis genes like Glucose transporter 1 (Glut1), Glucose transporter 3 (Glut3), hexokinase 2 and pyruvate kinase M (PKM) and represses their transcription. When Bcl6 expression vector was transfected into primary Th1 cells cultured in a high concentration of IL-2, Bcl6 was able to repress gene expression of glycolysis genes. These studies show that Bcl6 is able to repress glycolytic gene expression even if the culture conditions are favorable for the pathway.

PD-1, a marker of Tfh cells, was shown to alter T cell metabolic programming. Patsoukis et al showed that engagement of PD-1 with its ligand PD-L1 induced increased expression of genes involved in fatty acid oxidation. They showed that this metabolic programming aided the long-term survival of T cells (53).

Although the metabolic requirements for Tfh cell differentiation are well defined, the metabolic phenotype of human tonsillar cells has not been studied yet.

Figure 4

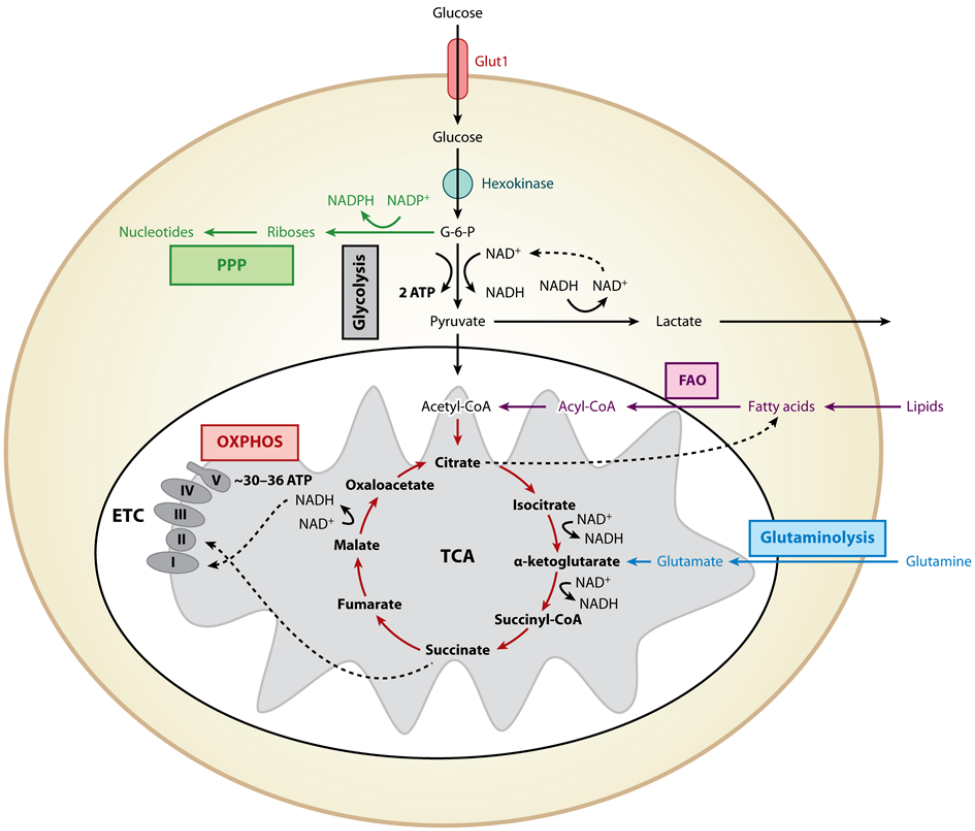


Figure 4. Metabolic pathways in immune cells

Major metabolic pathways in an immune cell are glycolysis, the TCA cycle and oxidative phosphorylation.

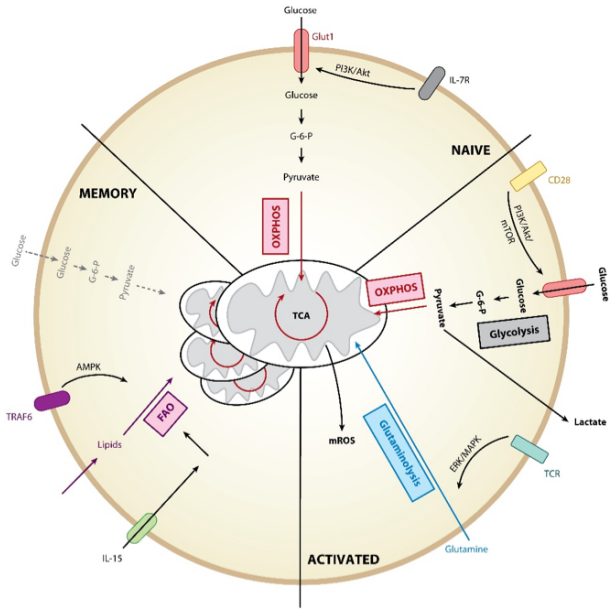
During glycolysis, Glucose taken up via Glucose transporter 1 (Glut1) is phosphorylated by hexokinase and sequentially processed to yield pyruvate and 2 ATPs. Glycolysis intermediates can be used to produce riboses via the pentose phosphate pathway. Pyruvate is redirected to the mitochondria and enters the TCA cycle after reduction to acetyl CoA. Fatty acid oxidation (FAO) can also fuel the TCA cycle by providing acetyl CoA. NADH produced by the TCA cycle is passed through the electron

transport chain and undergoes OXPHOS to yield 3-36 molecules of ATP. Pyruvate is converted to lactate that is excluded from the cells.

(modified from-MacIver NJ, Michalek RD, Rathmell JC.2013. Metabolic regulation of T lymphocytes. Annu Rev Immunol 31:259-283)

Figure 5

a)



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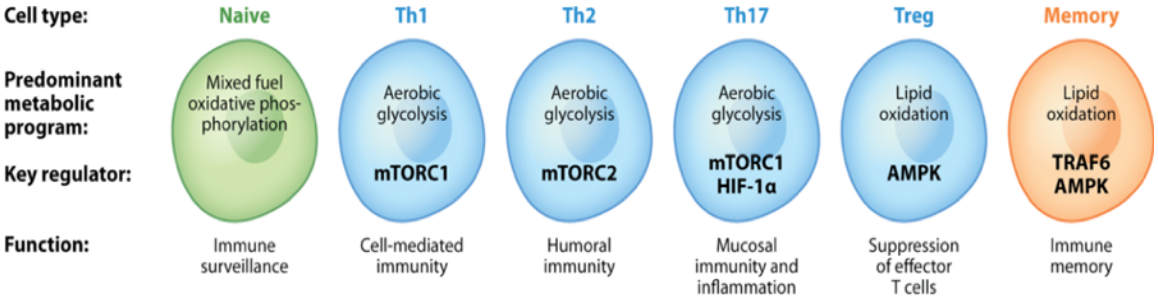


Figure 5. Metabolic phenotype of T cell stages and T helper cell subsets

a) Naïve T cells display a dependency on OXPHOS to meet their metabolic requirements. Upon activation, several signals including CD28 engagement trigger switch from OXPHOS to glycolysis. Activated T cells also have enhanced glutaminolysis. Upon resolution of the immune response, activated T cells convert to the quiescent memory T

cells which rely on fatty acid oxidation as the main source of energy. Memory T cells also have increased mitochondrial number.

b) mTOR kinases induce aerobic glycolysis in effector Th subsets- Th1, Th2 and Th17. AMPK induces FAO in Tregs and in memory T cells.

(modified from-MacIver NJ, Michalek RD, Rathmell JC.2013.Metabolic regulation of T lymphocytes. Annu Rev Immunol 31:259-283)

Figure 6

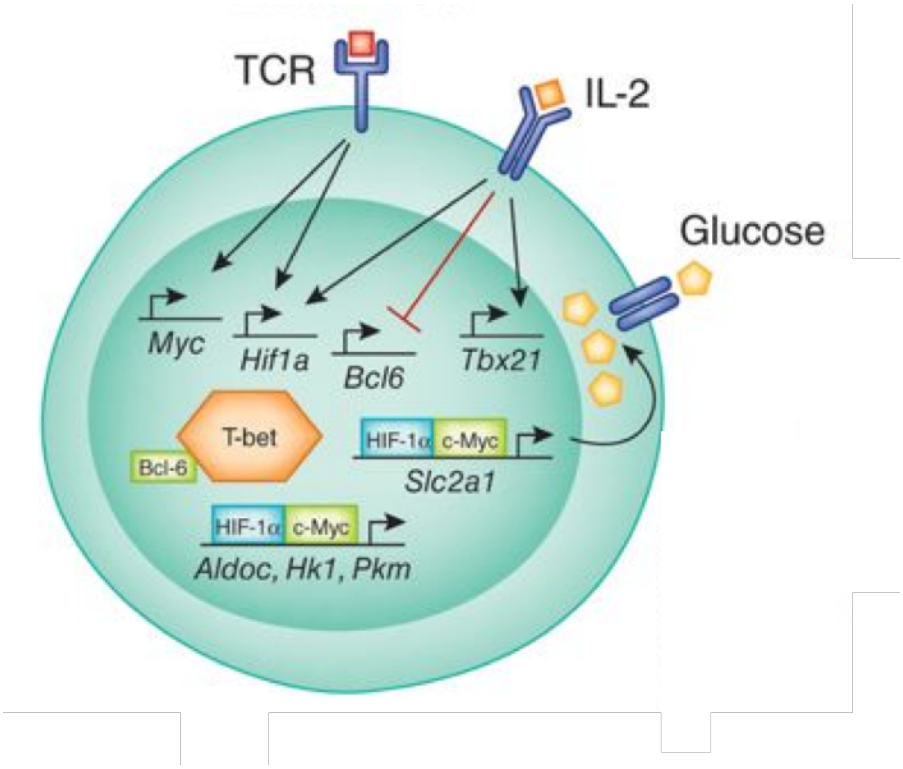


Figure 6. Bcl6 inhibits glycolysis when overexpressed in Th1 cells

IL-2 induces switch from fatty acid oxidation and oxidative phosphorylation to aerobic glycolysis by upregulating expression of cellular Myc (c-Myc) and Hypoxia-inducible factor 1–alpha (HIF1-α). TCR engagement also maintains aerobic glycolysis. Bcl6 inhibits T box containing protein expressed in T cells (Tbet) induced expression of glycolysis genes.

(Modified from – Kevin Man and Axel Kallies.2014. Bcl-6 gets T cells off the sugar, Nat Immunol, 15:904-905)

1.11 Hypothesis

Tfh cells have been shown to be major reservoirs of HIV (38). The frequency and absolute numbers of Tfh cells were shown to have expanded in patients chronically infected with HIV. The expansion of Tfh cell frequency may be attributed to their reduced susceptibility to HIV infection. The question whether this increase in Tfh cells in HIV patients is due to increased differentiation or reduced rate of HIV-induced cell death of infected cells is yet unanswered. Considering the unique niche of Tfh cells in the GCs, we hypothesized that Tfh cells have a unique metabolic phenotype that aids in their survival upon HIV infection. The four specific aims to test this central hypothesis are

Aim 1 – the metabolic phenotype of Tfh cells.

Aim 2 – metabolic requirements for human Tfh cell differentiation in-vitro

Aim 3 – effect of HIV infection on Tfh cell differentiation

Aim 4 – Survival of Tfh cells as a function of their metabolic phenotype.

Materials and Methods

2.1. Isolation of primary tonsillar T cells

Fresh tonsil tissue samples were obtained from the Indiana tissue bank in complacency with the IU IRB. Single cell suspensions were prepared from the tissue sample (how?). Mononuclear cells were isolated from the single cell suspension using Ficoll Hypaque density gradient centrifugation (GE Healthcare bioscience, Uppsala, Sweden). CD19+B cells and CD8+T cells were depleted from the mononuclear cells using positive magnetic separation beads. In brief, tonsillar mononuclear cells were incubated with CD19 and CD8 magnetic beads (Miltenyi, Bergisch Gladbach, Germany) on ice for 30 minutes. After 30 minutes of incubation, labelled CD19+B cells and CD8+T cells were depleted using a magnet. The enriched tonsillar T cells were counted using a hemocytometer and resuspended in RPMI supplemented with 10%FBS and 1% Penicillin, Streptamycin and L-glutamate (cRPMI).

2.2. Sorting of primary tonsillar T cells

Enriched tonsillar T cells were stained with CD4-FITC (SK3), CXCR5-APC (MU5UBEE) and PD-1-PECY7 (EH12.2H7). After 30 minutes of incubation at 4°C, cells were washed twice with PBS containing 2% bovine serum Fetal. Stained tonsillar T cells were gated on their expression of CD4. CD4 T cells were sorted into four populations based on their expression of CXCR5 and PD-1 using FACS ARIA sorter as shown in Figure 1a. The four populations were GC Tfh ($CD4^{+}CXCR5^{hi}PD\text{-}1^{hi}$), $CD4^{+}CXCR5^{-}PD\text{-}1^{+}$, $CD4^{+}CXCR5^{+}PD\text{-}1^{-}$ and $CD4^{+}CXCR5^{-}PD\text{-}1^{-}$.

2.3. RNA sequencing of tonsillar T cells

Total RNA was extracted from sorted tonsillar T cells using Pure Link RNA microkit (Invitrogen, Carlsbad, California) (please indicate resources of all reagents or kits) according to the kit protocol. RNA sequencing was performed at the Indiana University School of Medicine center for Medical Genomics. RNA concentration and quality was assessed using Agilent 2100 Bioanalyzer. Dual indexed strand specific cDNA library was prepared using KAPA mRNA Hyperprep kit. Quality and concentration of the prepared cDNA library was determined using Qubit and Agilent 2100 Bioanalyzer. Two hundred picomolar pooled libraries were utilized per flowcell for clustering amplification on cBot using HiSeq 3000/4000 PE Cluster kit and sequenced with 2X75 bp configuration on Illumina HiSeq 4000 using HiSeq 3000/4000 PE SBS kit. The sequenced libraries were mapped to the hg38 human genome using STAR RNA-seq aligner. Uniquely mapped sequencing reads were assigned to hg38 human genome using featureCounts. Differential expression analysis was performed using edgeR.

2.4. Isolation of naive human T cells and in-vitro Tfh differentiation

Buffy coat samples were obtained from healthy donors from Indiana Blood Bank (Indianapolis, IN). PBMCs were separated using Ficoll Hypaque (GE Lifesciences) density gradient centrifugation. Naive T cells were isolated from PBMCs using Human CD4 Naive T cell isolation kit (Biolegend, San Diego, CA). Isolated naive T cells were activated with human T cell activation beads (anti-CD3/anti-CD28; Life Technologies, Carlsbad, CA) for 24 hours. After 24 hours of activation, activated CD4 T cells were transferred into anti-CD3 coated wells and cultured in Glucose free complete RPMI (supplemented with 10% FBS and 100 U/ml of penicillin/streptomycin/glutamine)

supplemented with either 10 mM of D-Glucose or D-Galactose (Thermo Fisher, Waltham, Massachusetts) for 4 days. The cells were treated with 1 ng/ml IL-12 (Biolegend, San Diego, California) and 5 ng/ml TGF-β (Biolegend, San Diego, California)or 1 ng/ml IL-12 and 50 ng/ml Activin A (Minneapolis, Minnesota) for Tfh polarization or 10 ng/ml IL-12 and 1000 U/ml IL-2 (Biolegend, San Diego, California) for Th1 polarization.

2.5. HIV-1 NL4.3 Viral stock preparation

HIV-1 NL4.3 full length replication and infection competent chimeric DNA was obtained from NIH AIDS reagents program (NIAID, Bethesda, DC). The plasmids were transformed and expanded in One Shot Top10 chemically competent *E.coli.* Plasmids were extracted using Qiaprep midi kit. 18 micrograms of pNL4.3 was transfected into HEK293T cells in a T75 flask. Cell supernatant was collected 48 hours after transfection. The cell supernatant was filtered through a 0.2-micron filter and purified by centrifugation. Virus stock was titrated by measuring the concentration of HIV core protein using HIV-1 p24 ELISA kit (XpressBio, Frederick, MD)

2.6. *In-vitro* HIV infection

For infection of in-vitro differentiated T cells with HIV-1, the differentiated cells were incubated with HIV-1 NL4.3 at the concentration of 20 ng/ml HIV p24 at 37oC for 4 hours. After the incubation, cells were washed thrice and cultured in Glucose-free complete RPMI

For HIV infection before Tfh differentiation, the isolated naive Tfh cells were activated with human T cell activation beads (anti-CD3/CD28; Life Technologies, Carlsbad, CA) for 24 hours. The activated cells were incubated with HIV NL4.3 virus at

the concentration of 20 ng/ml of p24. After incubation with the virus at 37oC for 4 hours, the activated cells were transferred into anti-CD3 coated wells and cultured in Glucose-free cRPMI supplemented with 10 mM Glucose or Galactose for 4 days. The cells were treated with 1 ng/ml IL-12 and 5 ng/ml TGF-β or 1 ng/ml IL-12 and 50 ng/ml Activin A for Tfh polarization or 10 ng/ml IL-12 and 1000 U/ml IL-2 for Th1 polarization.

2.7. Antibodies and flow cytometry

Fluorophore-conjugated monoclonal antibodies against human CD4 (SK3,SK4), CXCR5 (MU5UBEE), PD-1 (EH12.2H7), HIV p24 (KC57), and BIRC5 (91630) were purchased from BioLegend (San Diego, CA) or BD Pharmingen (San Jose, CA). Fixable Viability Dye ef780 from eBiosciences (San Diego, CA) was used as a marker for live/dead cells.

Cells were subjected to surface staining first by incubating the cells with antibodies against human CD4, CXCR5 and PD-1 at 4°C for 30 minutes. After incubation, the cells were washed twice with PBS containing 2% FBS. The cells were then incubated with fixable viability dye ef780 diluted 1:1,000 in PBS at room temperature for 15 minutes. The cells were washed twice with PBS. For intracellular staining, the cells were permeabilized using BD Cytofix/CytoPerm Buffer for 30 minutes at room temperature. After fixing, the cells were washed twice with BD Perm wash buffer. The cells were then incubated with antibodies against HIV p24 or human BIRC5 at 4°C for 30 minutes. The cells were then washed twice with PBS containing 2% FBS. The cells were then analyzed using LSR4 flow cytometer. Flow cytometry data was analyzed using FlowJo software (FlowJo LLC, Ashland, Oregon

2.8. Determining mitochondrial mass of T cells

In-vitro differentiated T cells or isolated tonsillar T cells were subjected to cell surface staining and live/dead staining as previously described. The cells were then fixed with 2% PFA at room temperature for 30 minutes. The cells were washed twice with PBS containing 2% FBS. The cells were then incubated with Mito ID Green (Enzo Life Sciences, Farmingdale NY) diluted 1:2,000 in the assay buffer provided for 20 minutes at room temperature. The cells were then immediately acquired on the LSR4 flow cytometer.

2.9. Seahorse assay

Tonsillar T cells were sorted as described above. The cells were cultured overnight in cRPMI. The sensor cartridge was hydrated overnight with 200 µl per well of sterile water. After overnight hydration, the sensor cartridge was calibrated using 200 µl per well of Agilent seahorse XF calibrant and incubating for 1 hour at 37°C in a non-CO2 incubator. The seahorse XF96 cell culture miniplates were coated with Cell Tak (Corning, Corning New York) at the concentration of 22.4 ng/ml. The plate was incubated for 15 minutes at 37°C in a non-CO2 incubator. After 15 minutes, the wells were washed with sterile water. The cells were counted using a hemocytometer and resuspended in RPMI (without FBS and antibiotics) at the concentration of 1 million cells/ml. 200 µl of cell suspension was added to each well so that each well had 0.2 million cells. The cell culture miniplates were incubated at 37°C in a non-CO2 incubator. The compounds- Oligomycin, Antimycin A, Rotenone and Carbonyl cyanide-4 (trifluoromethoxy) phenylhydrazone (FCCP) were purchased from Cayman biologicals (Ann Arbor, Michigan). The compounds were diluted to the concentrations of 100 µM

for Oligomycin, 500 μM for FCCP and 100 μM each for Antimycin A and Rotenone. The compounds were added to the sample injection ports as instructed by the manufacturer so that the final concentrations of the compounds in the assay were - 10 μM for Oligomycin, Antimycin A and Rotenone and 50 μM for FCCP. The assay was performed on a seahorse Xf96 extracellular flux analyzer (Agilent, Santa Clara, California). The results were normalized and analyzed using the wave software (Agilent, Santa Clara, California).

2.10. Statistical analysis

Statistical analysis was performed using Graphpad prism 6 software (GraphPad software, San Diego, California). Student's t test was used for comparison between two groups.

Results

3.1. RNA sequencing revealed differential expression of metabolism genes

To unravel the difference in metabolic gene expression between follicular and non-follicular CD4 T cells, we performed transcriptome sequencing on human tonsillar CD4 T cells. CD4 T cells were isolated from tonsils of three HIV-1-negative donors who had undergone otolaryngoscopic surgery. Tonsillar CD4 T cells were then sorted based on their expression of CXCR5 and PD-1 into four subpopulations including CXCR5-PD-1-, CXCR5-PD-1+, GC Tfh (CXCR5hiPD-1hi) and CXCR5+PD-1 (Figure a). Sorted cells were subjected to RNA extraction for RNA-seq analysis. Transcriptome analysis revealed that there was difference in the expression of metabolic genes compared between GC Tfh and CXCR5-PD-1- cells. GC Tfh cells showed reduced expression of genes associated with glycolysis when compared to CXCR5-PD-1- cell population. Instead, GC Tfh cells had increased expression of genes involved in fatty acid oxidation and gluconeogenesis (Figure 7c). Gene ontology analysis revealed that genes associated with fatty acid oxidation were the most enriched group in GC Tfh cells (Figure 7d). These results indicate that Tfh cells are less reliant on glycolysis for their energy requirements.

Figure 7

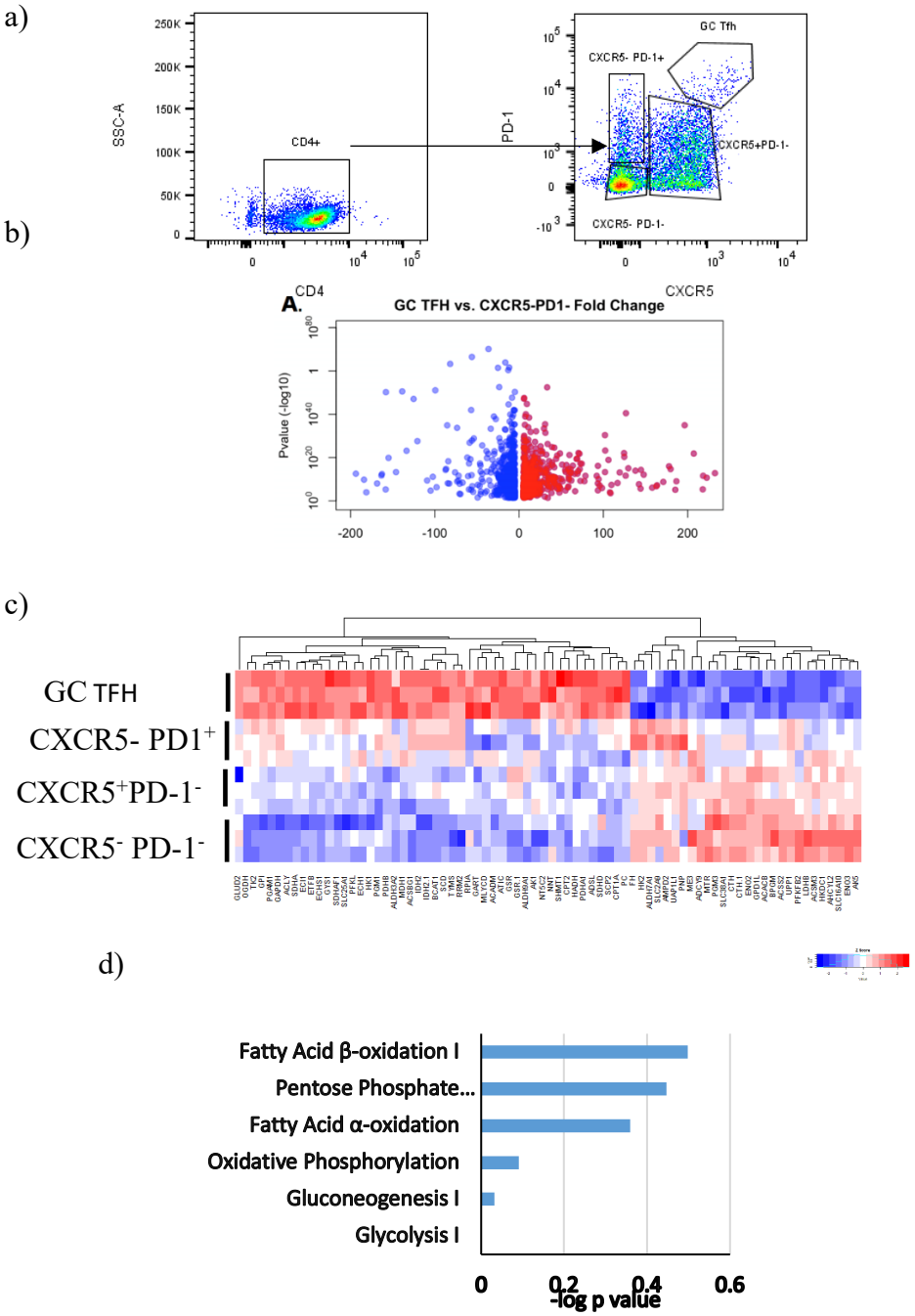


Figure 7. RNA sequencing revealed differential expressions of metabolism genes

a) Representative flow plots showing the gating strategy for the sorting of tonsillar CD4T cells. Tonsillar CD4T cells were sorted into four populations based on their expression of CXCR5 and PD-1.

b) Volcano plot showing differential gene expression between GC Tfh and CXCR5-PD-1- tonsillar T cells.

c) Heatmap showing differential gene expression of metabolic genes between GC Tfh and other tonsillar CD4 T cell subsets.

d) KEGG pathway analysis of differentially expressed metabolic genes in GC Tfh cells.

3.2. Seahorse assay revealed oxidative metabolic phenotype of Tfh cells

RNA sequencing data revealed that Tfh cells have lower expression of glycolytic genes when compared to non-Tfh cells. To confirm the metabolic phenotype of Tfh cells, I performed Seahorse extracellular flux assay with sorted tonsillar CD4T cell subsets. Seahorse extracellular flux assay measures oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) of live cells at intervals of approximately 5-8 minutes. The OCR is a measure of mitochondrial respiration and ECAR is indicative of the rate of glycolysis. The Seahorse mito-stress test was performed to determine the metabolic phenotype of tonsillar CD4 T cells and to analyze the parameters of mitochondrial function. In the mito-stress test, several respiration modulating compounds are added to the assay to reveal mitochondrial functional dynamics.

Oligomycin inhibits ATP synthase (complex V), and is injected first in the assay following basal measurements. It reduces electron flow through the ETC, resulting a reduction in mitochondrial respiration or OCR. This decrease in OCR is linked to cellular ATP production. Carbonyl cyanide-4 (trifluoromethoxy) phenylhydrazone (FCCP) is an uncoupling agent that collapses the proton gradient and disrupts the mitochondrial membrane potential. It is the 2nd injection following Oligomycin. As a result, electron flow through the ETC is uninhibited, and oxygen consumption by complex IV reaches the maximum. The FCCP-stimulated OCR can then be used to calculate spare respiratory capacity, defined as the difference between maximal respiration and basal respiration. Spare respiratory capacity is a measure of the ability of the cell to respond to increased energy demand or under stress. The third injection is a mixture of rotenone, a complex I inhibitor, and antimycin A, a complex III inhibitor. This combination shuts down

mitochondrial respiration and enables the calculation of non-mitochondrial respiration driven by processes outside the mitochondria.

Seahorse mito-stress test revealed that GC Tfh cells have higher basal oxygen consumption rate when compared to other tonsillar CD4 T cell subsets (Figure 8a, b). GC Tfh cells also had higher spare respiratory capacity, maximal oxygen consumption rate and ATP production from respiration as compared to other tonsillar CD4 T cell subsets (Figure 8c, d, e)

These results verify that Tfh cells have reduced reliance on glycolysis and exhibit oxidative metabolic phenotype.

Figure 8

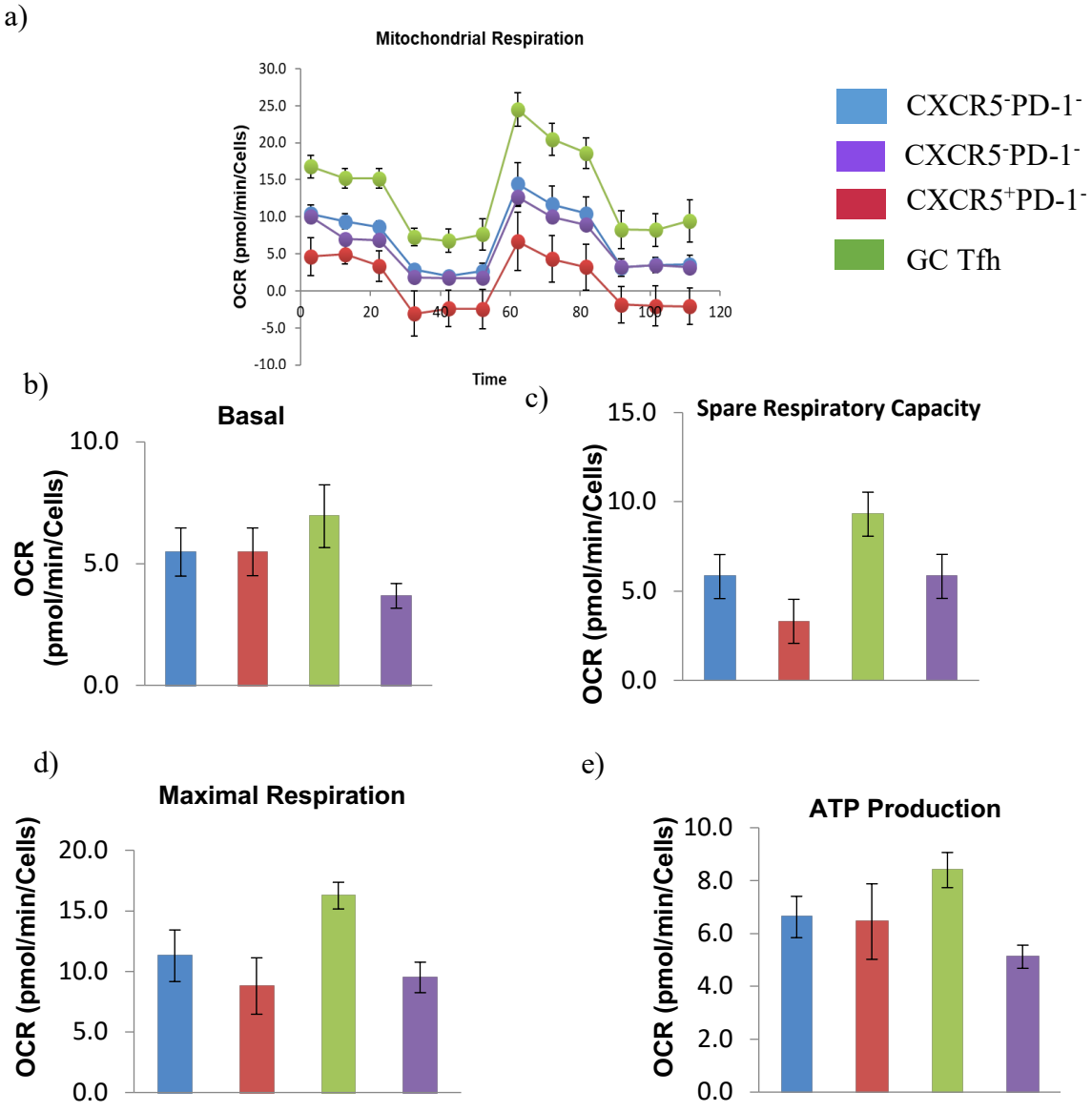


Figure 8. Seahorse assay revealed oxidative metabolic phenotype of Tfh cells

a) Graph showing Oxygen consumption rate over time and compound injection strategies.

b) Basal oxygen consumption rates for tonsillar CD4 T cell subsets.

c) Spare respiratory capacity of tonsillar CD4 T cell subsets.

d) Maximal respiration of tonsillar CD4 T cell subsets.

e) ATP production associated with respiration for tonsillar CD4 T cell subsets

Data is represented as mean ± SEM of 5 replicates.

3.3. Galactose yielded higher frequency of Tfh cells

To determine if glycolysis is necessary for in-vitro differentiation of Tfh cells, we differentiated Tfh cells in either Glucose- or Galactose-containing medium. After 4 days of differentiation, we determined Tfh cell frequency using flow cytometry.

The results show that Galactose medium yielded higher frequency of Tfh cells compared to Glucose medium (Figure 9a, 9b). The absolute number of $CXCR5^{+}PD\text{-}1^{+}$ cells was also higher in Galactose medium (1,770 ± 1.9 SEM, n=) compared to Glucose medium (755 ± 0.8 SEM, n=) (Figure 9c).

I compared the two previously described methods for in-vitro differentiation of Tfh cells (24, 25). Without addition of polarizing cytokines, we observed minimal frequency of Tfh cells (Figure 9d). The results found no significant difference in Tfh cell frequency when we compared the Tfh yield between the two methods ($p = 0.1$, n=4) (Figure 9d)

Figure 9

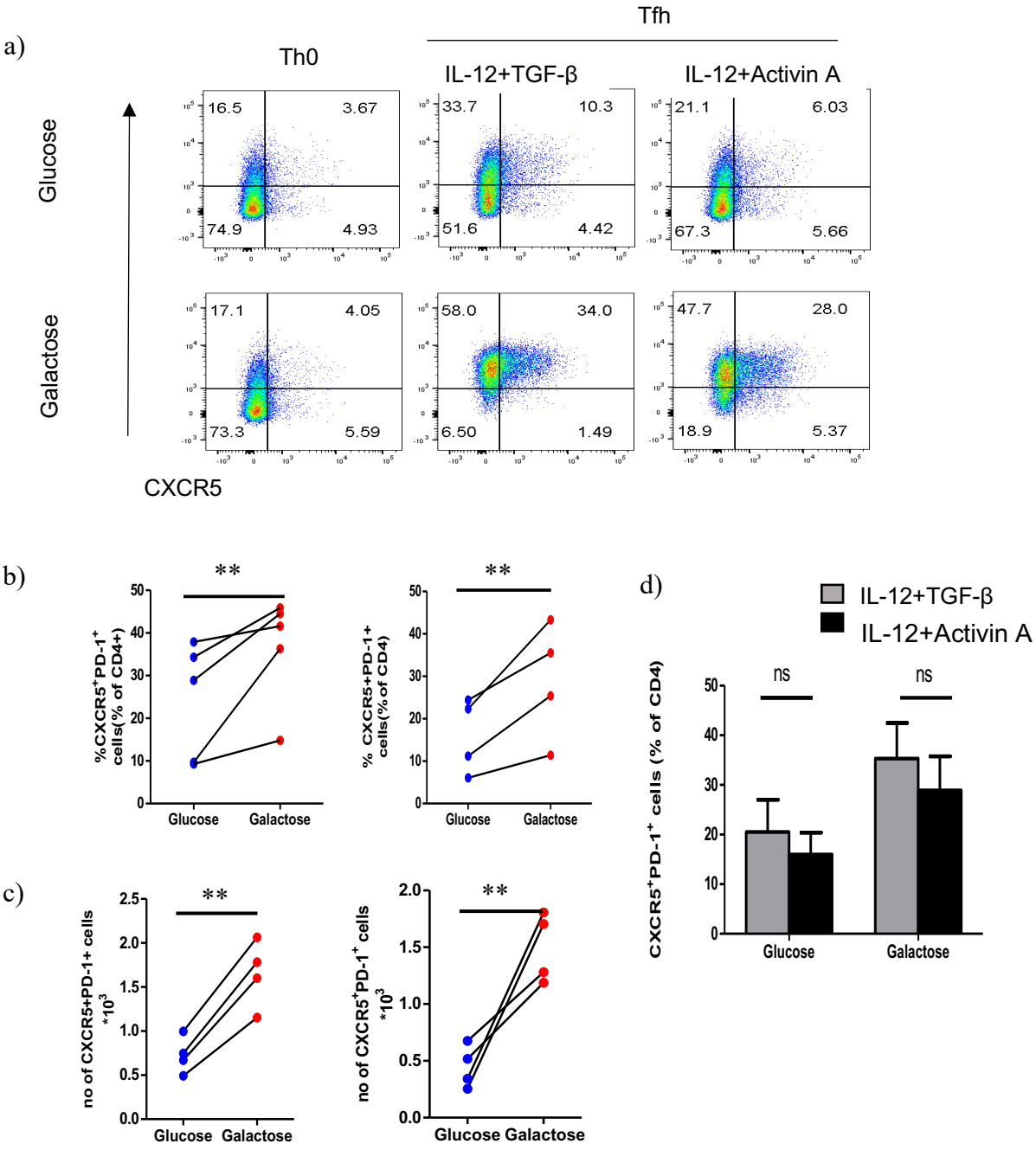


Figure 9. Galactose yielded higher frequency of Tfh cells

a) Representative flow plots showing Tfh cell frequencies upon in-vitro differentiation in Glucose or Galactose containing medium.

b) Graphical representation of Tfh cell frequencies upon in-vitro differentiation in glucose and galactose containing medium.

c) Graphical representation of Tfh cell numbers upon in-vitro differentiation in glucose or galactose containing medium.

d) Graphical representation of the comparison between the two approaches for in-vitro Tfh differentiation.

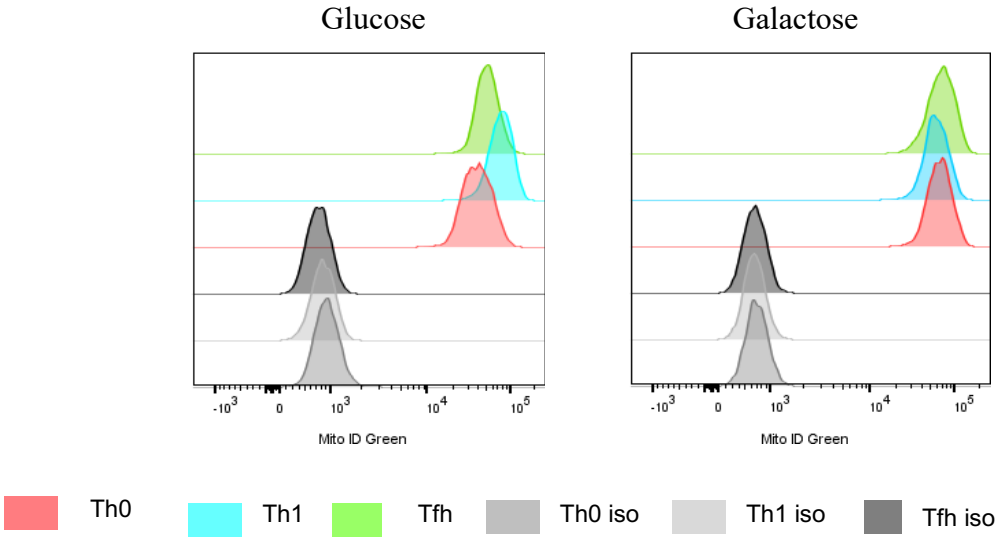
Data is represented as mean ± SEM of five (IL-12+TGF-β) or four (IL-12+Activin A) independent experiments. Student's t test was used to analyze data. **$p \leq 0.01$, ns-not significant.

3.4. Mitochondrial mass was comparable between Tfh and non-Tfh cells

To determine if the increased use of OXPHOS in Tfh cells was due to increase in mitochondrial mass, I first differentiated naïve human CD4 T cells into either Tfh, Th1 or non-polarized (Th0) cells and then stained the cells with MitoID Green. MitoID Green stains the mitochondria independent of the mitochondrial membrane potential and is used to measure the mitochondrial mass (54). I observed no difference in MitoID Geen fluorescence intensity when I compared between Tfh, Th1 and Th0 cells (Figure 10a and b).

Figure 10

a)



b)

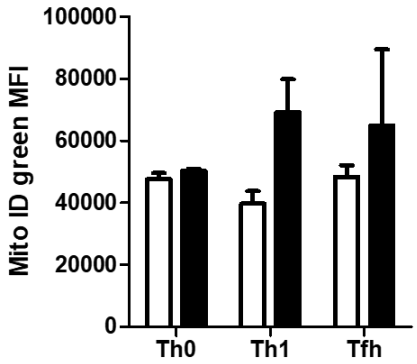


Figure 10. Mitochondrial mass was comparable between Tfh and non-Tfh cells

a)Representative flow plots showing mean fluorescence intensity (MFI) of MitoID green in in-vitro differentiated Th0, Th1 and Tfh cells.

b)Graphical representation of MitoID Green MFI in in-vitro differentiated Th0, Th1 and Tfh cells.

Data is represented as mean ± SEM of 2 independent experiments.

3.5. HIV Infection reduced differentiation of Tfh and non-Tfh cells

I hypothesized that HIV infection affects Tfh cell differentiation dependent on the cell's metabolism. For the study, we activated naïve T cells for 24 hours. After activation, the cells were incubated with HIV-1 NL4.3 strain equivalent to 20 ng/ml p24 for 4 hours. The cells were then incubated with Tfh polarizing cytokines for 4 days and then Tfh cell frequency was assessed by flow cytometry. I found that in uninfected condition, Galactose yielded higher frequency of Tfh cells. HIV infection abrogated in-vitro Tfh differentiation in both Glucose and Galactose condition (Figure 11a). The reduction in Tfh cell frequency after HIV infection was significantly higher in Glucose condition compared to that in Galactose condition (Figure 11b). HIV infected cells were quantified by staining with antibodies against HIV p24. I found that HIV p24+ cell frequency was higher in Galactose than in Glucose condition (Figure 11c, 4d)

These results indicate that though there is a higher frequency of HIV infected cells in the Galactose condition, it did not result in significant reduction of Tfh cell frequency as compared to Glucose condition.

Figure 11

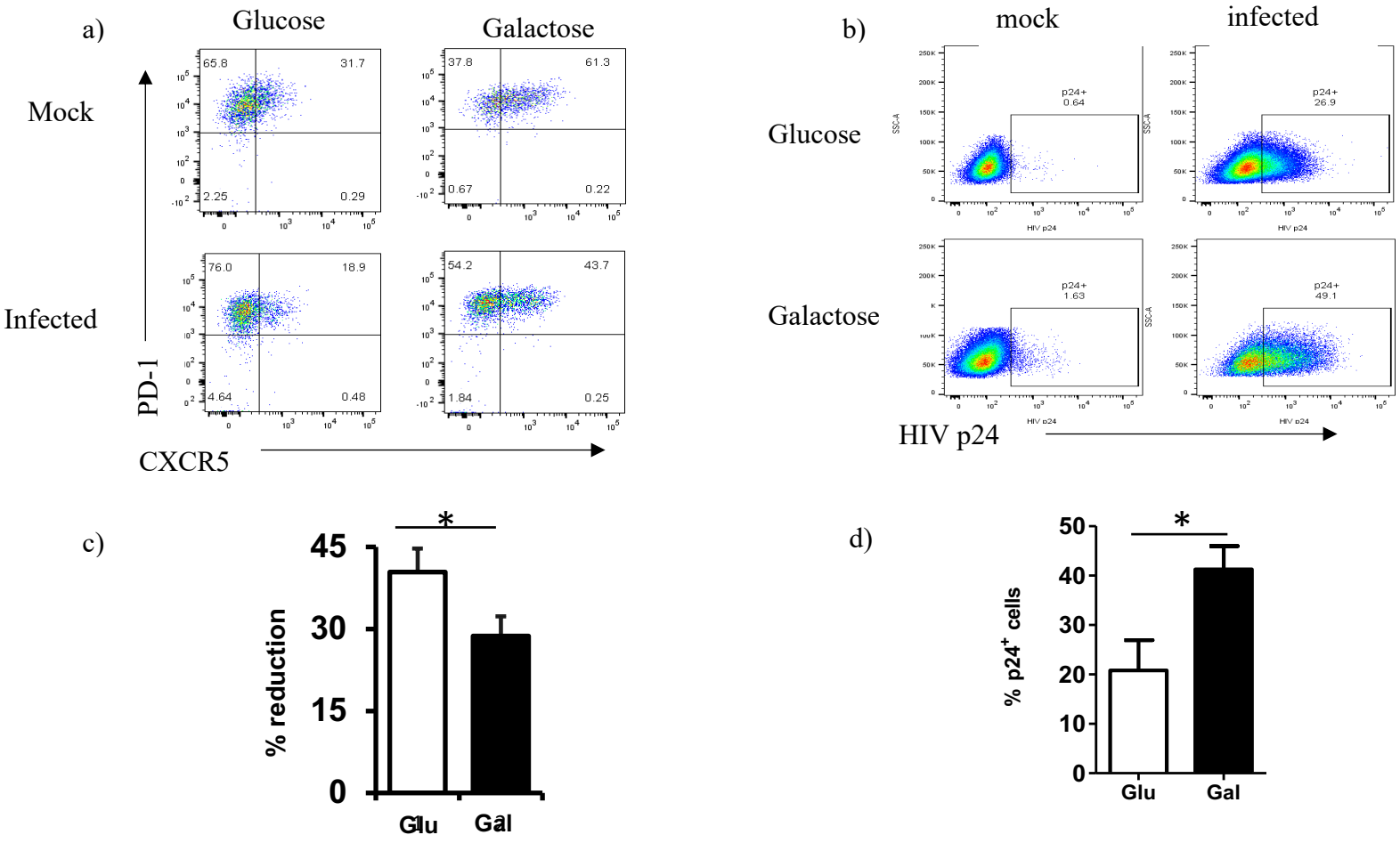


Figure 11. HIV Infection reduced differentiation of Tfh and non-Tfh cells

a) Representative dot plots depicting Tfh cell frequencies after in-vitro differentiation upon HIV infection.

b) Representative dot plots showing HIV p24 expression in infected Tfh cells.

c)Graphical representation of the reduction of Th frequency after HIV infection in Glucose and Galactose medium

d)Graphical representation of the HIV p24+ T cells in Glucose and Galactose medium.

Data is represented as mean ± SEM of 4 independent experiments. Student's t test was used for statistical analysis. *$p<0.05$

3.6. Ex-vivo tonsillar CD4 T cell subsets expressed CD69 but were resistant to HIV infection unless activated

I wanted to examine whether the survival of primary tonsillar T cells upon HIV-1 infection was dependent on their metabolic phenotype. I isolated tonsil tissue CD4 T cells from HIV-negative donors. The isolated CD4 T cells were either activated with anti-CD3/anti-CD28 beads or mock-activated with PBS and followed by infection with HIV-1 NL4.3 equivalent to 100 ng/ml p24. The cells were cultured in Glucose or Galactose containing medium for 4 days. After four days the cells were analyzed for viability and HIV p24. I observed that tonsillar CD4 T cells could be divided into five populations based on their expression of CXCR5 and PD-1 (Figure 12a) as described previously. When I looked at the activation markers such as CD25, CD38, CD69 and HLA-DR expression on tonsillar T cells I found that there was minimal expression of activation markers CD25, CD38 and HLA-DR. Whereas I found that there was high frequency of tonsillar CD4 T cell subsets expressed CD69 (Figure 12b). I next wanted to examine whether CD69 expression alone was sufficient to allow HIV-1 infection. The results showed that un-activated tonsillar CD4 T cells were resistant to HIV-1 infection, although they were CD69-positive (Figure 12c).

Figure 12

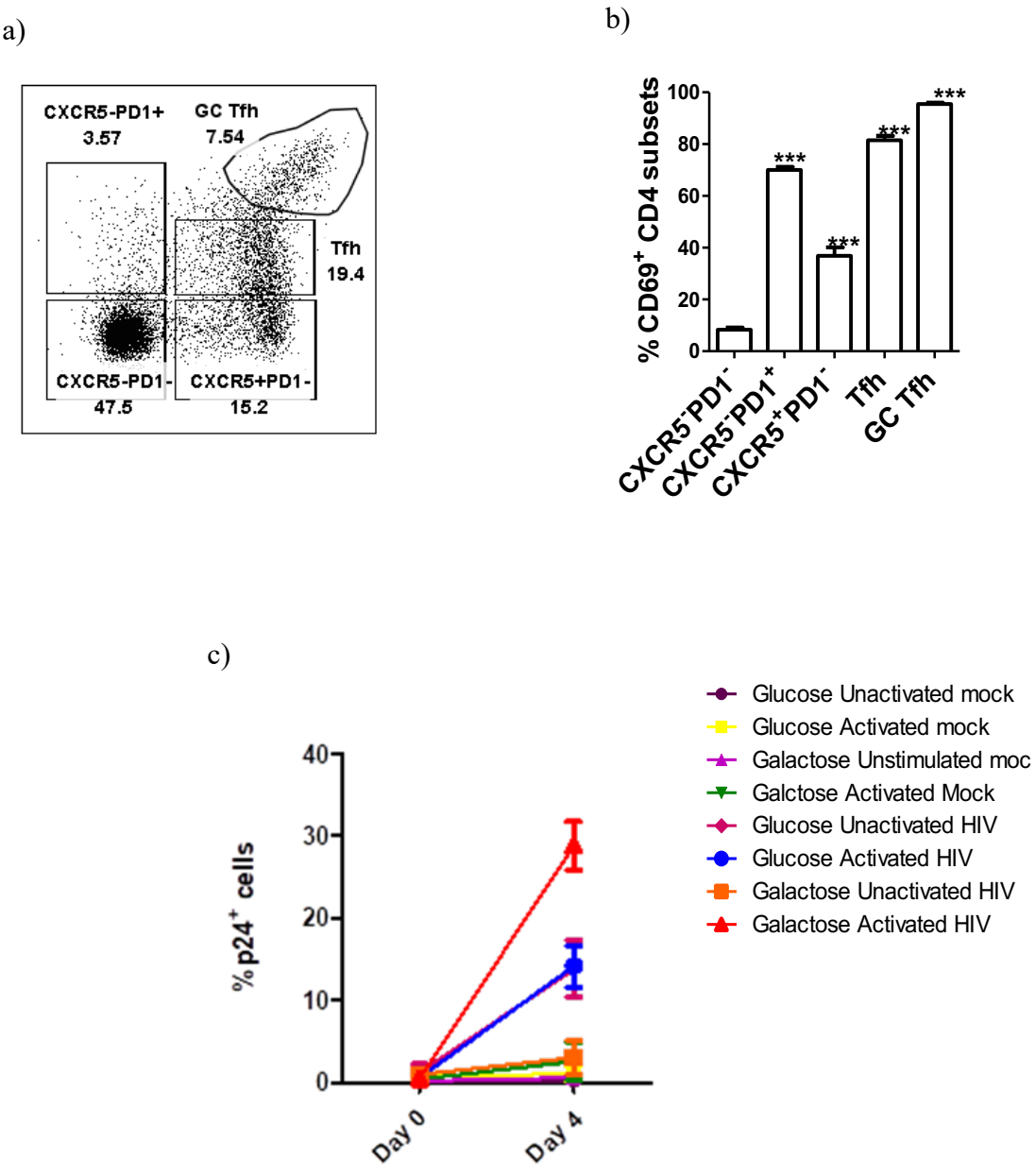


Figure 12. Ex-vivo tonsillar CD4 T cell subsets expressed CD69 but were resistant to HIV infection unless activated

a) Representative dot plots showing tonsillar CD4T cell subsets based on their expression of CXCR5 and PD-1.

b) CD69 expression on tonsillar CD4 T cell susets.

c) Graphical representation showing HHIV-1 p24+ T cells among the tonsillar CD4 T cell subsets.

Data is represented as mean ± SEM of three independent experiments. Student's t test was used for statistical analysis. ***$p \leq 0.001$

3.7. HIV infected ex-vivo tonsillar T cells survive better in Galactose medium

I next assessed survival of infected tonsillar CD4 T cells in Glucose versus Galactose medium. I found that upon HIV infection, the frequency of viable tonsillar CD4 T cells was higher in Galactose medium compared to Glucose medium (Figure 13b). When I looked at the frequency of HIV-1-infected cells, I observed that the frequency of HIV p24-positive cells was higher in Galactose medium compared to Glucose medium (Figure 13c). These results indicate that HIV-infected tonsillar CD4 T cells survived better in Galactose medium compared to Glucose medium despite of having higher frequencies of HIV-infected cells.

Figure 13

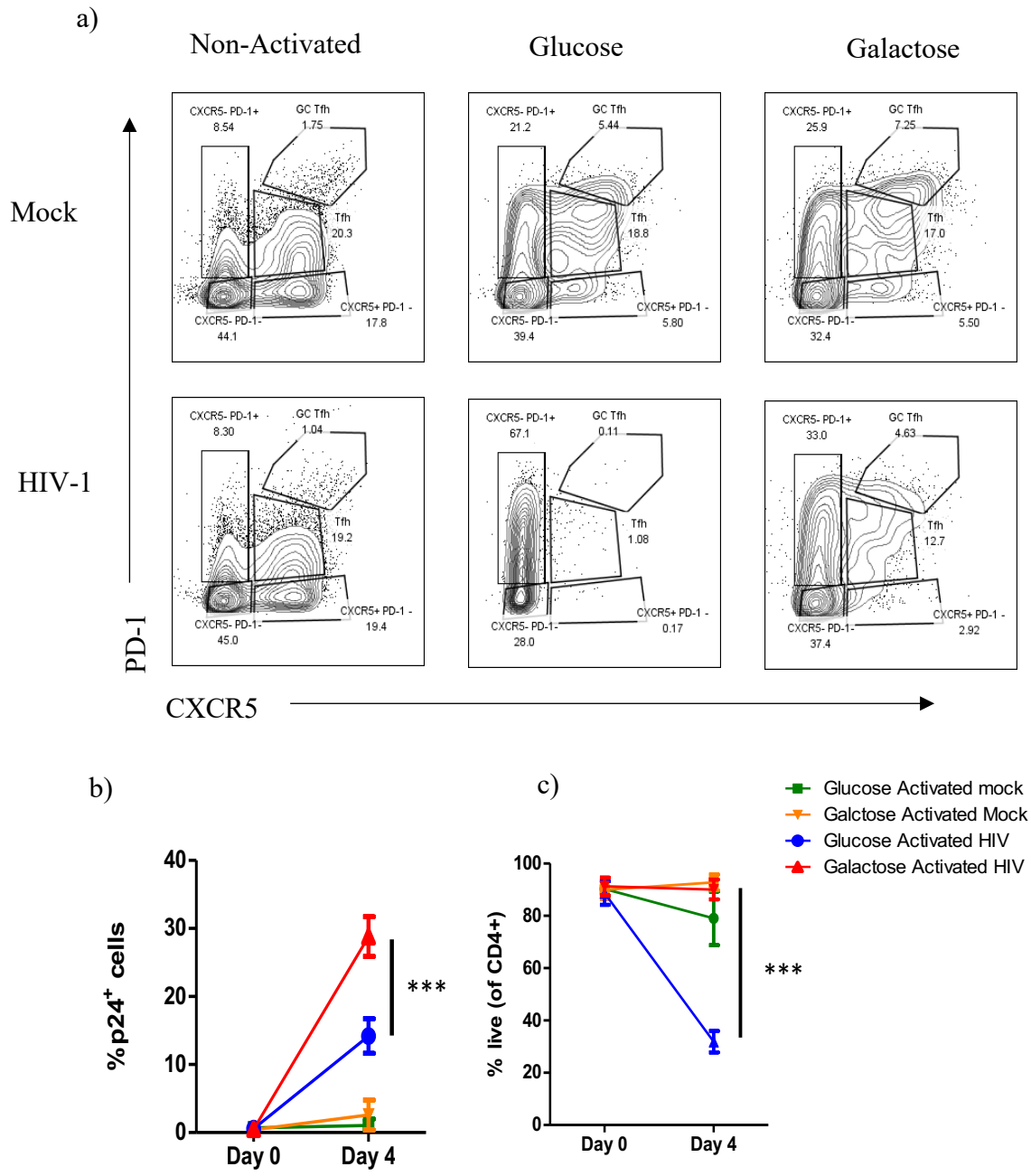


Figure 13. HIV infected ex-vivo tonsillar T cells survive better in Galactose medium

a) Representative dot plots showing frequencies of tonsillar CD4 T cell subsets cells upon HIV-1 infection

b) Graph showing HIV-1 $p24^{+}$ CD4 T cells on day0 and day 4 after HIV-1 infection

cultured in Glucose or Galactose medium.

c) Graph showing frequency of live HIV-1 infected tonsillar CD4T cells on day 0 and day 4 post HIV-1 infection cultured in Glucose or Galactose medium.

Data is represented as mean ± SEM of 3 independent experiments. Student's t test was used for statistical analysis. ***$p \leq 0.001$

3.8. BIRC5 expression was upregulated in CD4 T cells cultured in Galactose medium upon HIV infection

A recent study has shown that the expression of Baculoviral IAP repeat containing protein 5 (BIRC5) is upregulated in CD4 T cells latently or productively infected with HIV. Inhibition of BIRC5 resulted in reduced frequency of HIV infected CD4 T cells with intact HIV DNA (58). The transcriptome sequencing analysis revealed that expression of several anti-apoptotic genes was higher in GC Tfh cells (Figure 14a). Interestingly the expression of BIRC5 was about 23-fold higher in GC Tfh compared to $CXCR5^{-}PD\text{-}1^{-}$ cells. I studied whether expression of BIRC5 was dependent on the cells' metabolism. I cultured HIV infected tonsillar T cells in Glucose or Galactose medium and measured the expression of BIRC5 using flow cytometry. I found that expression of BIRC5 was higher in Galactose medium even in the mock infected cells. The expression of BIRC5 was further upregulated on HIV infected tonsillar T cells cultured in Galactose medium compared to Glucose. The results suggest that the enhanced survival of infected Tfh cells in Galactose medium can be partly explained by the upregulation of BIRC5.

Figure 14

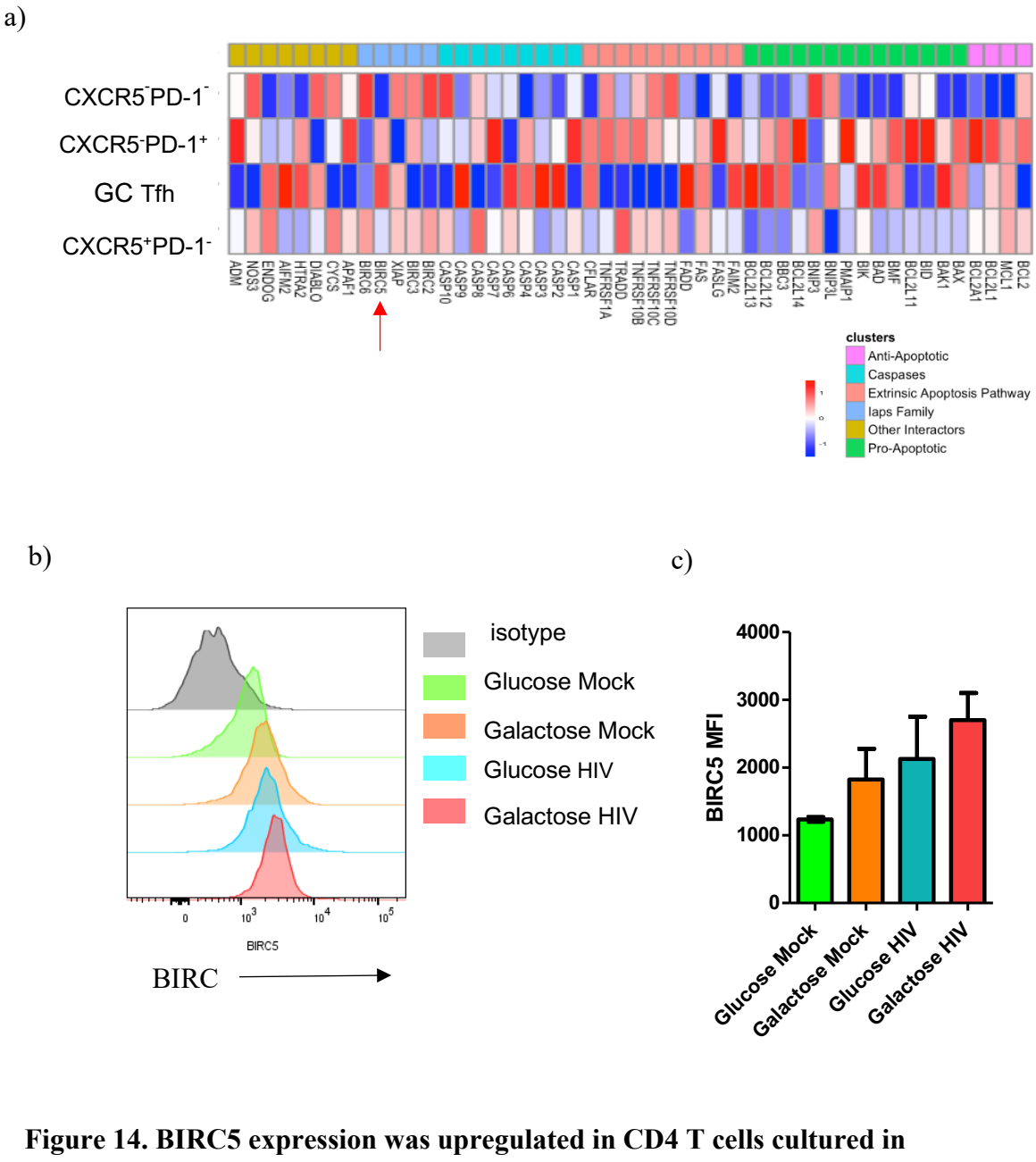


Figure 14. BIRC5 expression was upregulated in CD4 T cells cultured in Galactose medium upon HIV infection

a) Heatmap showing differential gene expression of apoptosis related genes among tonsillar CD4 T cells.

b) Representative flow-cytometry plots showing BIRC5 MFI on HIV-1 infected tonsillar CD4 T cells 4 days post infection.

c) Graph showing BIRC5 MFI on HIV-1 infected tonsillar T cells on day 4 post infection. Data is represented as mean ± SEM of 3 independent experiments. Student's t test was used for statistical analysis.

Discussion

Tfh cells have been shown to be susceptible to HIV infection. Tfh cells serve as major reservoirs of latent and productive HIV infection even upon ART. In contrast to the fate of other CD4 T cell subsets, Tfh cell frequency was shown to have increased during HIV infection. The expansion of Tfh cells during HIV infection may be due to increased differentiation of Tfh cells or due to their increased survival.

Transcriptome analysis of tonsillar CD4 T cells revealed differential expression of metabolism genes between Tfh and non-Tfh cells. Specifically, Tfh cells had higher expression of genes involved in fatty acid oxidation and gluconeogenesis whereas non-Tfh cells had higher expression of glycolysis genes. Gene set enrichment analysis showed that Tfh cells displayed a phenotype that resembles naïve T cells. Based on the gene expression analysis, the hypothesizes was that Tfh cells have unique metabolism that drives their differentiation and survival upon HIV infection.

I first studied the metabolic pathway necessary for Tfh cell differentiation. I differentiated activated CD4 T cells into Tfh cells in the presence of Glucose or Galactose to drive the cells to use either glycolysis or OXPHOS, respectively. The results indicate significantly higher frequency of Tfh cells when the cells were differentiated in Galactose containing medium. The results reflect the studies by Xie et al (55). They showed that Glucose depletion mediated induction of 5'- Adenosine monophosphate activated protein kinase (AMPK) induces expression of Bcl6 in activated CD4 T cells in mice. The studies together indicate that glycolysis is not necessary for Tfh differentiation in-vitro.

I next studied the effects of HIV infection and metabolism on Tfh differentiation. I found that HIV infection reduced Tfh differentiation in both Glucose and Galactose medium. This reduction in Tfh cell frequency was significantly higher in Glucose medium.

Several cell intrinsic and extrinsic factors could contribute to the decreased differentiation of Tfh cells upon HIV infection. IL-12 is crucial for Tfh differentiation in humans. Marshall et al have shown that HIV-1 infection caused dysfunction of IL-12 signaling in PBMCs. They showed that HIV infected PBMCs have reduced expression of the IL-12 receptor β chain (IL-12R β)(56). TGF-β levels are increased during HIV infection but immune cells are refractory to the effect of TGF-β. George et al showed that the expression of transforming growth factor B and mothers against decapentaplegic homolog 5 (SMAD5), a TGF-β signal transduction molecule is downregulated during SIV infection (57). Depletion of cytokines that drive Tfh polarization during HIV infection may contribute towards reduced Tfh differentiation.

The next aim was to next study if the survival of infected Tfh cells was dependent on their metabolism. The results showed that HIV infected tonsillar Tfh cells survive better upon HIV infection when cultured in Galactose medium. Transcriptome analysis revealed that Tfh cells express higher levels of the anti-apoptotic molecule-BIRC5. BIRC5 protects cells against extrinsic and intrinsic pathways of apoptosis by directly inhibiting the activation of caspase 9. Expression of BIRC5 and its upstream regulator - tumor necrosis factor receptor superfamily member 4 (TNFRSF4, also known as OX40), were shown to have increased during HIV infection (58). OX40 plays a critical role in long-term survival of memory T cells (59). Kuo et al also show that OX40+ CD4 T cells

isolated from HIV infected patients had higher clonally expanded HIV-1 DNA compared to $OX40^-$ CD4 T cells (58). These results indicate that $OX40^+$ CD4 T cells act as viral reservoirs. The increased BIRC5 expression in HIV-infected Tfh cells in Galactose condition may thus play a role in their enhanced survival and contribute to maintain viral reservoir in Tfh cells. Whether the oxidative phenotype of Tfh cells is an effect of increased BIRC5 expression is unclear and needs further studying. Strikingly, pharmacological inhibition of BIRC5 reduced the number of infected cells harboring intact viral DNA. Therefore, BIRC5 is a viable target for elimination of viral reservoirs in Tfh cells.

In addition to cell intrinsic factors, cell extrinsic factors such as the concentration of ART drugs in the lymph nodes and cytotoxicity of HIV specific CD8 T cells in the lymph nodes also play important roles in the survival of HIV infected Tfh cells.

Fletcher et al have shown that lymphatic tissues have lower concentration of ART drugs compared with peripheral blood (60). The low concentration of ART drugs in the lymph nodes is the cause for persistent viral replication observed in the lymph nodes.

Li et al show that SIV-specific CD8 T cells were excluded from the GCs. They also showed that the frequency of follicular CD8 T cells was inversely correlated with viral RNA-positive cells (61). Therefore, in addition to targeting BIRC5, strategies to increase concentration of ART drugs in the lymph nodes and improving CD8 cytotoxicity in the GCs are critical for elimination of infected Tfh cells.

Future directions

The results indicate that Tfh cell differentiation does not require glycolysis. HIV infection reduces Tfh differentiation in Glucose and Galactose medium but this reduction is significantly higher in Glucose medium. The results show that HIV infected Tfh cells survive better in Galactose medium. This enhanced survival may be due to upregulation of the anti-apoptotic BIRC5 in Galactose medium.

Cells cultured in Galactose medium show increased OXPHOS. Several pathways including fatty acid oxidation and amino acid metabolism contribute towards increased OXPHOS. Studies with fatty acid oxidation inhibitors or amino acid metabolism inhibitors are required to further dissect the pathway important for Tfh cell differentiation. Inhibiting glycolysis using 2 deoxy-Glucose will further confirm the contribution of glycolysis in Tfh differentiation.

Tfh cells are characterized by their expression of the transcription repressor Bcl6 and by their ability to secrete IL-21. I plan to study the expression of Bcl6 and IL-21 in the in-vitro differentiated Tfh-like cells to validate their Tfh phenotype.

Metabolic phenotype of a cell can be measured by quantifying the extracellular proton concentration and their oxygen consumption rate. The seahorse extracellular flux assay measures the proton concentration as an indicator of the rate of glycolysis and oxygen consumption rate as the rate of OXPHOS. Seahorse extracellular flux assays with sorted tonsillar CD4T cells and in-vitro differentiated Tfh cells to define their metabolic phenotype should be repeated to get a solid understanding.

Studies have shown that the GC microenvironment is hypoxic (62). Currently, we

culture the cells in normoxic conditions. Culturing Tfh cells under hypoxic conditions will closely mimic their *in-vivo* niche. Culturing the Tfh cells under hypoxic conditions will lead to better understanding their metabolism under physiological conditions.

The main role of Tfh cells is to provide help to B cells for antibody production. Several studies have shown that the humoral response in dysregulated during HIV infections indicating defects in Tfh cell functions. I plan to study how metabolism contributes to Tfh cell function. For this purpose, we will incubate Tfh cells with autologous B cells in Glucose or Galactose medium and measure the quantity and isotype of the secreted antibodies.

The results show that infected Tfh cells have higher levels of BIRC5. BIRC5 plays a role in the survival of memory T cells. We plan to use the BIRC5 inhibitor, 1-(2-Methoxyethyl)-2-methyl-4, 9-dioxo-3-(pyrazin-2-ylmethyl)-4, 9-dihydro-1H-naphtho [2, 3-d] imidazolium bromide (YM155) to study its effect on HIV infected Tfh cells in context of their metabolism. YM155 suppresses transcription from the BIRC5 promoter. Studies have shown that YM155 is specific against BIRC5 and has no effect on other inhibitors of apoptosis family members (IAPs). If upregulation of BIRC5 is important in survival of infected Tfh cells, inhibition of BIRC5 should reduce the survival of the infected Tfh cells.

CD8 T cells in the follicle kill HIV infected Tfh cells. To determine whether metabolism affects CD8 T cell response to infected HIV Tfh cells. We plan to co-culture HIV infected Tfh cells in Glucose or Galactose medium labelled with radioactive chromium isotope with autologous CD8 T cells.

A better understanding of mechanism contributing towards maintaining Tfh cells as HIV reservoirs will help in devising strategies for elimination of HIV reservoirs. Eradication of cellular HIV reservoirs will ultimately lead to an HIV cure.

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Yanyan Xing , Nicole Shepherd , Jie Lan , Wei Li , Sushmita Rane , Samir K. Gupta Shanxiang Zhang , Jun Dong , Qigui Yu
Brain, Behavior, and Immunity 65 (2017) 161–172

Primary human B cells at different differentiation stages exhibit distinct susceptibilities to Vaccinia virus binding and infection.
Nicole shepherd,Wei Li, Jie Lan, Wei Li, Sushmita Rane, Quigui Yu
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